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The emerging roles and functions of circular RNAs and their generation

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Abstract

Circular RNAs (circRNAs) are closed long non-coding RNAs, in which the 5′ and 3′ termini are covalently linked by back-splicing of exons from a single pre-mRNA. Emerging evidence indicates that circRNAs are broadly expressed in mammalian cells and show cell type- or tissue-specific expression patterns. Importantly, circRNAs have been shown to participate in regulating various biological processes. Functionally, circRNAs can influence cellular physiology through various molecular mechanisms, such as serving as a decoy for microRNAs or RNA-binding proteins to modulate gene expression or translation of regulatory proteins. The biogenesis of circRNAs is known to be tightly regulated by *cis*- (intronic complementary sequences) and/or trans-factors (splicing factors) that constitute a cell- and context-dependent regulatory layer in the control of gene expression. However, our understanding of the regulation and function of circRNAs is still limited. In this review, we summarize the current progress in elucidating the functional roles, mechanisms and biogenesis of circRNAs. We also discuss the relationship between regulation and formation of circRNAs.

Keywords: Circular RNAs (circRNAs), Splicing factor, Micro RNAs (miRNAs), Long non-coding RNAs (lncRNAs)

Background

As most eukaryotic genes are interrupted by non-informational introns, nascent RNA transcripts typically undergo splicing to remove introns, after which the exons are fused colinearly to form mature linear RNA transcripts (Fig. 1). Splicing is a highly regulated process, which may generate multiple mature RNA isoforms from a given gene, and these isoforms may exhibit different functions, cellular locations or regulatory roles [1]. Over 95% of human genes have alternatively spliced isoforms [2], the expression of which is determined by both trans-regulatory factors and *cis*-regulatory elements, including splicing factors and their binding motifs.

Circular RNAs (circRNAs) are generated by a specific type of splicing called back-splicing, wherein the 5′ terminus of a pre-mRNA upstream exon is non-colinearly spliced with the 3′ terminus of a downstream exon (Fig. 1). CircRNAs are predominantly found in the cytoplasm, and the lack of a 5′ cap and 3′ tail make the circular molecules more resistant to RNase degradation compared to their linear cognates [3]. The existence of mammalian circRNAs was first reported in 1979 by Hsu, who observed the molecules in the cytoplasm of HeLa and other mammalian cells by electron microscopy [4]. However, due to technical limitations, only a few specific circRNAs were identified throughout the next two decades, and the potential functions of circRNAs remained unclear [5–9]. With the development of next generation sequencing, alongside the publication of complete genome sequences and the advance of bioinformatics technology, researchers have discovered that the expression of circRNAs in mammals is often conserved across species, and shows tissue and cell specificity. The expression level of some circRNAs can be higher than the linear cognates [10–14].

Importantly, Memczak et al. and Hansen et al. first demonstrated that the circular isoform of human antisense to cerebellar degeneration-related protein 1 RNA (*CDR1as*) is functional in neural development, and this striking observation launched the nascent field of circRNA research [15, 16]. The number of published circRNA studies has grown exponentially in the following years, making circRNAs some of the most notable molecules in RNA biology. Several databases (e.g., circBase, circNet, Circ2Traits,

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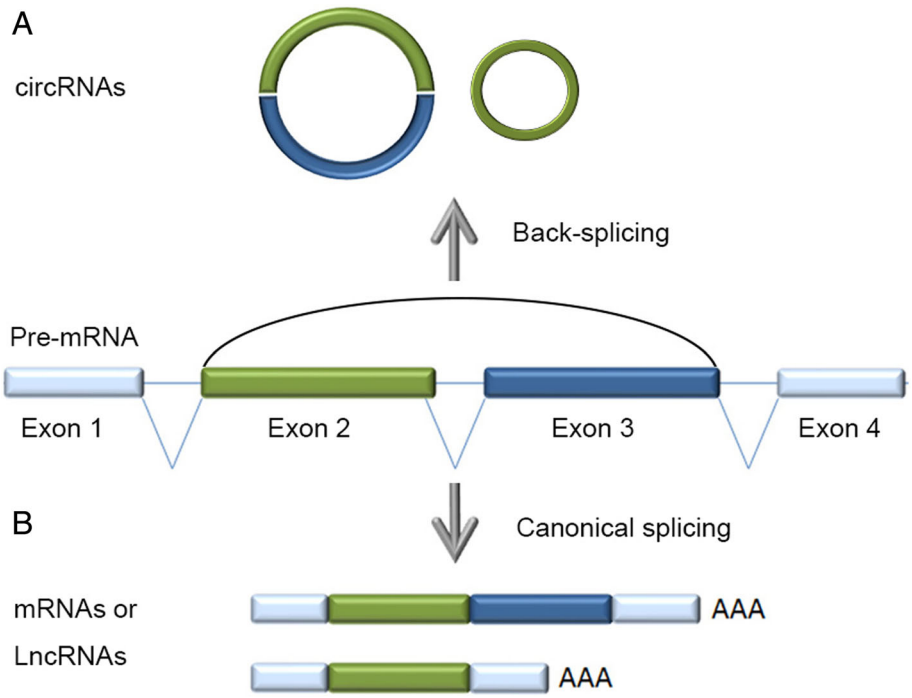

Fig. 1 Back-splicing and canonical splicing of a single pre-mRNA. (**a**) The single pre-mRNA can be back-spliced with the 5′ terminus of upstream exon 2 ligated to the 3′ terminus of downstream exon 3 to generate a circRNAs. (**b**) Otherwise, the exons of the pre-mRNA can be joined colinearly by canonical splicing to form mRNAs or lncRNAs

exoRBase, and CSCD) have been created to curate circRNAs from different species and provide further information about association with diseases, cellular locations and other non-coding RNAs. The availability of this information speeds up the exploration of circRNA functions and underlying mechanisms by which circRNAs exert functions [17–22].

Although most circRNAs are spliced from protein coding pre-mRNAs, circRNAs are usually categorized as long non-coding RNAs (lncRNAs). Similar to other lncRNAs, circRNAs can serve as RNA or protein decoys to regulate gene expression. The most well-known type of circRNA interaction is with microRNAs (miRNAs). Individual circRNAs can harbor multiple miRNA binding sites to act as a "sponge" and inhibit activity of one or multiple miRNAs. CircRNAs also form complexes with proteins to regulate the cell cycle [23] or translation [24], or to serve as intercellular signaling molecules in released exosomes [25, 26]. Interestingly, some circRNAs may encode functional peptides, as demonstrated in recent work showing that circRNAs were able to be translated in vitro and in vivo [27–30].

CircRNA formation competes with formation of linear cognates, indicating that the canonical spliceosome has some involvement in back-splicing [31, 32]. Short intronic repeats or Alu elements promote circRNA formation in cis [33–35], whereas RNA binding proteins (e.g., splicing factors) play important roles in regulating circRNA formation in trans [32, 36–38]. Despite many exciting advances in circRNA biology, the number and identities of molecules involved in circRNA biogenesis and how regulatory networks control circRNA function remained largely unclear. In this review, we summarize the known functions of circRNAs in mammalian cells and the mechanisms by which circRNAs exert these functions. We also survey the factors that regulate circRNA formation and discuss the relationship between function and formation of circRNAs.

CircRNAs regulate cell proliferation

Accurate and precise control of the cell cycle is important during normal cellular responses to environmental cues. Dysregulation of the cell cycle in neural stem cells may cause megalencephaly or microcephaly [39], while a lack of cell cycle control in somatic cells can promote cancer progression [40]. A growing number of circRNAs have been reported to regulate proliferation through effects on signaling pathways, transcription factors and cell cycle checkpoint regulators. Two major pathways that regulate cell proliferation and are affected by circRNAs include MAPK/ERK and PI3K/AKT. In the MAPK/ERK pathway, growth factors (e.g., FGF) bind to receptor tyrosine kinases (e.g., FGFR), which then phosphorylate MAPK to activate ERK and promote cell proliferation. *CDR1as* and *circHIPK3* were shown to promote EGFR receptor expression in

colorectal cancer (CRC) and esophageal squamous cell carcinoma (ESCC) [41, 42], while *circWDR77* enhanced FGF2 ligand expression in vascular smooth muscle cells [43](Fig. 2a). In the PI3K/AKT pathway, ligands (e.g., insulin) bind to receptor tyrosine kinases, which activate PI3K to phosphorylate AKT and promote cell proliferation. In hepatocellular carcinoma (HCC) and glioblastoma, *CDR1as* and *circNT5E* were found to promote cell proliferation by increasing PI3K expression [44, 45] (Fig. 2b). CircRNAs also regulate the WNT/β-catenin pathway to promote proliferation. For example, knockdown of *circHIPK3* was shown to decrease WNT2 ligand and FZD4 receptor expression, which decreased the level of nuclear β-catenin and hampered retinal endothelial cell proliferation [46]. Moreover, *circZFR* potentiated β-catenin expression in HCC and promoted proliferation [47] (Fig. 2c). In addition, *circHIPK3* can promote proliferation in human cell lines, probably through upregulation of IL6R expression [48]. Transcription factors and cell cycle checkpoints are also found to be targets of circRNA regulation. For instance, disruption of *circTCF25* and *circRNA_100290* in cancer cells downregulates CDK6 expression, affecting the proliferation of bladder cancer and osteosarcoma cells [49, 50] (Fig. 2d). Moreover, circRNA *hsa_circ_0008039* is reported to increase E2F3 expression, inducing S-phase transition and promoting proliferation of breast cancer cells [51] (Fig. 2d). On the other hand, circRNAs may also inhibit cell proliferation. Ectopic expression of *circITCH* and *circZFR* upregulates PTEN expression, which inhibits proliferation of bladder cancer and HCC cells [52, 53] (Fig. 2d). Furthermore, *circITCH* promotes ITCH and CBL expression, which inhibits cell proliferation by downregulating the WNT/β-catenin pathway [54, 55] (Fig. 2c). Similarly, *hsa_circ_0002052* induces APC2 expression, which promotes β-catenin degradation to inhibit osteosarcoma cells proliferation [56] (Fig. 2c). In another example, *circFOXO3* is shown to interact with and sequester P21 and CDK2 in the cytoplasm, attenuating cell cycle progression [23] (Fig. 2d).

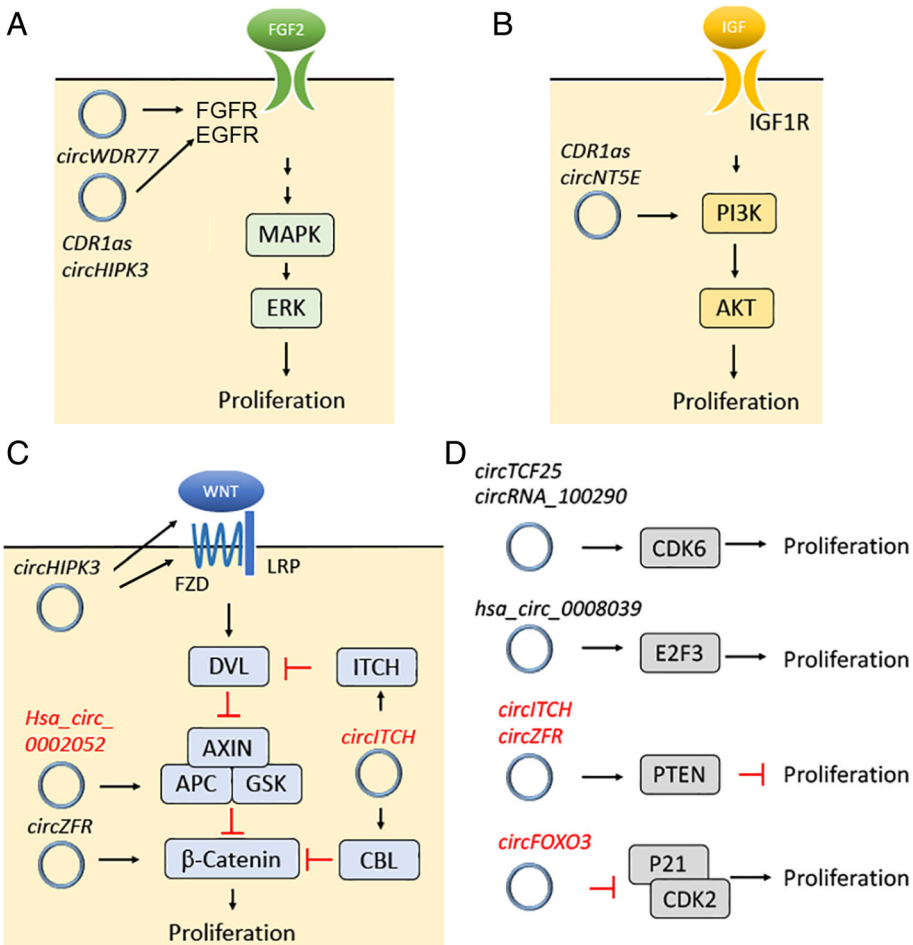

Fig. 2 CircRNA regulates cell proliferation. CircRNA regulates cell proliferation through multiple factors, including (**a**) FGF2 and EGFR in MAPK/ERK pathway, (**b**) PI3K in PI3K/AKT pathway, (**c**) WNT2, FZD4, ITCH, CBL, APC2, and β-catenin in WNT/β -catenin pathway, and (**d**) CDK6, E2F3, PTEN, P21 and CDK2 that regulate cell cycle. CircRNAs promote or inhibit cells proliferation are labeled by black and red, respectively

Together, these reports demonstrate that circRNAs can regulate cell proliferation through a variety of different mechanisms.

CircRNAs regulate epithelial-mesenchymal transition (EMT) and cancer progression

EMT is highly regulated during development to ensure correct localization of differentiated cells at the proper times. The improper activation of EMT is frequently found in the early stages of cancer progression and causes cancer cell migration and invasion. EMT is mainly induced by TGF-β family ligands, which stimulate the phosphorylation and nuclear translocation of R-SMADs and co-SMADs to activate SNAI, bHLH and ZEB transcription factors [57]. Accumulating evidence suggests that circRNAs contribute to cancer progression by regulating the EMT process. *circMYLK* was found to act on the TGF-β signaling pathway by increasing TRAF4 expression in PC-a cells to attenuate degradation of the TGF-β receptor and promote EMT [58]. *circRNA_0084043* also promoted EMT by upregulating SNAI expression in melanoma cells [59]. Similarly, *circIRAK3*, *circNASP*, *circMAN2B2* and *circSHKBP1* respectively promoted FOXC1, FOXF1, FOXK1 and FOXP1 expression, all of which upregulated SNAI expression in cancer cells [60–63]. CircRNAs have also been shown to inhibit EMT. For example, *circSMAD2* upregulated TRIM33, which trapped SMAD4 to block the TGF-β signaling cascade in HCC cells [64]. Additionally, disruption of *circFOXO3* decreased FOXO3 expression, which promoted EMT in non-small-cell lung carcinoma (NSCLC) [65]. These results are summarized in Fig. 3.

CircRNAs regulate pluripotency and early lineage differentiation

Pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are able to differentiate into many cell types in our body or in culture. In human ESCs and iPSCs, disruption of *circBIRC6* and *circCOROC1* negatively affects pluripotency maintenance, whereas expression of *circBIRC6* and *circCORO1C* promotes pluripotency reprogramming of iPSCs. Further exploration of the regulatory mechanisms reveals that *circBIRC6* inhibits the activity of miR-34a and miR-145, preventing downregulation of pluripotency transcription factors NANOG, OCT4 and SOX2 [36]. These results suggest that circRNAs play roles in pluripotency maintenance and differentiation. In line with this finding, a recent study of global circRNA expression during human ESCs differentiation showed that *circRMST* and *circFIRRE* are enriched in differentiated hESCs, suggesting that certain circRNAs are associated with ESC differentiation [66]. Further, circRNAs are also involved in somatic stem cell differentiation. For example, *CDR1as* is shown to regulate neural development in zebrafish and osteoblastic differentiation of periodontal ligament stem cells (PDLSC), while ectopic expression of *circFGFR4*, *circSVIL* and *circZNF609* induce myoblast differentiation [15, 67–71]. Interestingly, *circZNF609* may also promote myoblast differentiation through the actions of an encoded small peptide [27].

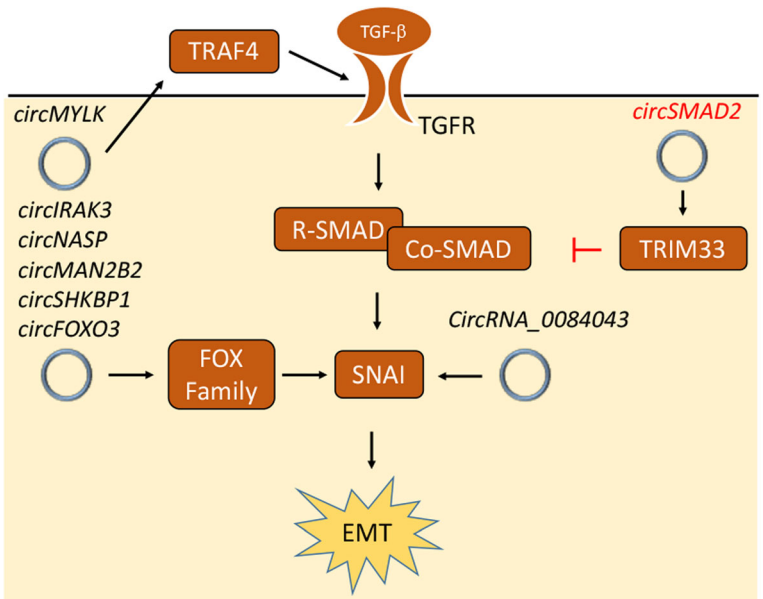

Fig. 3 CircRNA regulates EMT and cancer progression. CircRNA regulates EMT and cancer progression through multiple factors, including TRAF4, TRIM33, SNAI, FOXC1, FOXF1, FOXK1, FOXO3 and FOXP1 in TGF- β pathway. CircRNAs promote or inhibit EMT are labeled by black and red, respectively

Other circRNA functions

CircRNAs have also been shown to also regulate unique functions of specialized cells. For example, *SRY* is a well-known sex determining gene for testis development, which encodes both linear and circular RNAs [5, 72]. In addition to being translated into SRY protein, the RNA product of *SRY* may also serve as a sponge for miR-138 [15]. Another known example of circRNA function in specialized cells is β-cells in pancreatic islets, which produces and secretes insulin. Both *CDR1as* and *circHIPK3* were found to promote insulin secretion from β-cells [73, 74]. In the immune system, *circZC3H4* and *circHECTD1* can promote the activation of alveolar macrophages, which stimulates fibroblast proliferation and migration [75, 76]. In the nervous system, disruption of *circHIPK2* and *circHECTD1* inhibits astrocyte activation, which may be beneficial during stroke recovery [77, 78]. Few circRNAs were reported to regulate apoptosis. In two contrasting examples, *hsa_circ_0043256* induces apoptosis by increasing ITCH in NSCLC cells, but *circGRB10* inhibits apoptosis by increasing ERBB2 in nucleus pulposus (NP) cells [79, 80]. Finally, circRNAs also play important roles in several human diseases. It was previously reported that *circANRIL* interacts with PES1 to regulate rRNA maturation and promotes the development of atherosclerosis [81]. Additionally, *circDLGAP4* is found to relieve damage from ischemic stroke in brain tissue [82]. The ectopic expression of *circVMA21* decreases intervertebral disc degeneration of NP cells [83], while *circZNF609* regulates retinal neurodegeneration in retinal ganglion cells and vascular dysfunction in endothelial cells [84, 85].

The mechanisms of circRNA functions

CircRNAs as miRNA sponge

The most prominent function of circRNAs is its action as a miRNA sponge to regulate target gene expression by inhibiting miRNA activity. One circRNA can regulate one or multiple miRNAs through multiple miRNA binding sites in the circular sequence. For example, the first identified functional circRNA, human *CDR1as*, has 74 miR-7 binding sites, 63 of which were found to be conserved in one other species. *CDR1as* is shown to be enriched in neural tissues, and knocking out *CDR1as* expression in mouse or zebrafish impairs midbrain development through miR-7 dysregulation [15, 71, 86]. In human cells, knockdown of *CDR1as* expression also dysregulates miR-7 expression and affects insulin secretion, cell proliferation and the pathobiology of myocardial infarction [42, 44, 70, 71, 73, 87–90]. The testis-specific circular *SRY* controls sex determination in mammals [5]. Circular *SRY* has 16 miR-138 binding sites and was shown to interact with miR-138 and AGO2 in HEK293 cells, suggesting that *SRY* acts as a miR-138 sponge [16]. *circHIPK3* has 18 miRNA binding sites for nine different miRNAs, among which the inhibition of miR-124 activity promotes cell proliferation in HCC and gallbladder cancer cells [48, 91, 92]. *circHIPK3* also targets miR-338-3p to regulate insulin secretion by β-cells [74]. It is not surprising that circRNA has been shown to regulate different downstream genes through different miRNAs. For example, *circITCH* sequesters miR-214 and miR-22-3p to promote ITCH and CBL expression, thereby regulating the WNT/β-catenin pathway [54, 55]. *circITCH* also increases PTEN and RASA1 expression (components of PI3K/AKT and MAPKERK pathways) by targeting miR-17/224 and miR-145, respectively [53, 93]. Interestingly, circRNA may even target different miRNAs to exert opposite functions in different cells. For example, *circZFR* targets miR-130a/107 to upregulate PTEN and inhibits gastric cancer cell proliferation, but targets miR-1261/4302/3619 to promote HCC proliferation [47, 52, 94, 95]. Many circRNAs have been shown to function through sponging miRNAs, and we have summarized the known instances in Table 1.

CircRNAs as protein decoys

In addition to interacting with miRNAs, circRNAs can serve as protein decoys to influence cellular functions. For example, *circFOXO3* is shown to trap CDK2/p21 and HIF-a/ID1 in the cytoplasm, which blocks cell cycle progression and induces cell senescence, respectively [23, 96]. *circFOXO3* also promotes the interaction between MDM2 and P53, which decreases the P53 protein level and induces apoptosis [97]. In breast cancer cells, *circDNMT1* activates autophagy by promoting P53 and AUF1 nuclear translocation [98]. In vascular tissue, *circANRIL* sequesters PES1 to impair rRNA maturation, resulting in apoptosis [81]. In glioblastoma multiforme cells, *circSMARCA5* inhibited migration by stimulating splicing factor SRSF1 to modulate expression of the SRSF3 isoform [99]. In breast cancer cells, *circMTO1* sequesters TRAF4, preventing Eg5 activation and decreasing cell viability [100]. In primary cardiomyocytes, *circAmotl1* interacts with PDK1 and AKT1, which induced the phosphorylation and nuclear translocation of AKT1, reducing apoptosis to promote cardiac repair [101]. In HeLa cells, *circPABPN1* recruits HuR to suppress its interaction with PABPN1 mRNA, which led to reduced PABPN1 translation [24]. These protein decoy functions of circRNAs are summarized in Table 2.

Translatable circRNAs

Although circRNAs are considered to be lncRNAs with low protein coding potential, it has been shown that circRNAs containing an internal ribosome entry site (IRES) or N^6-methyladenosine modification and can be translated into peptides in vitro and in vivo [28, 102–105]. Notably, many circRNAs contain the start codon of cognate mRNAs associated with ribosomes [29]. These findings indicate that circRNAs are sometimes able to be translated. Pamudurti et al. further shows that endogenous *circMbl3* produces a

Table 1 circRNAs that function as miRNA sponges

CircRNA	Biological functions	miRNA	Targets	Cell type	Ref
CDR1as	Neural development	miR-7/− 671	Fos	Neural tissue	[15, 42, 44, 70, 71, 73, 87–90]
	Insulin secretion	miR-7	Pax6, Myrip	Islet cells	
	Myocardial infarction	miR-7	PARP, SP1	Cardiomyocytes	
	Promotes proliferation	miR-876-5p	MAGE-A	ESCC	
	Anti-oncogenic	miR-7/−135a	P21	Bladder cancer	
	Promotes proliferation	miR-7	CCNE1, PIK3CD, EGFR	NSCLC	
	Promotes proliferation/metastasis	miR-7	HOXB13	ESCC	
	Osteoblastic differentiation	miR-7	GDF5	PDLSC	
SRY	Sex-determining	miR-138		Testis	[16]
circBIRC6	Pluripotency maintenance	miR-34a/−145	NANOG, OCT4, SOX2	hESCs, iPSCs	[36]
circHIPK3	Promotes proliferation	miR-124/− 152/−193a/− 29a/−29b/− 338/− 379/− 584/− 654	IL6R, DLX2	Cancer tissues	[41, 46, 48, 74, 91, 92, 114]
	Inhibits cancer progression	miR-558	HPSE	Bladder cancer	
	β-cell function	miR-124-3p/338-3p	Slc2a2, Akt1, Mtpn	β-cells	
	Promotes proliferation	miR-30a-3p	FZD4, WNT2, VEGF-C	Retinal endothelial cells	
	Promotes proliferation/migration	miR-124	AQP3	HCC	
	Promotes proliferation/migration	miR-7	FAK, IGF1R, EGFR	CRC	
	Promotes proliferation	miR-124	ROCK1, CDK6	Gallbladder cancer	
circWDR77	Promotes proliferation	miR-124	FGF2	VSMC	[43]
circNT5E	Promotes cancer progression	miR-422a	PIK3CA, NT5E	Glioblastoma	[45]
circZFR	Promotes cancer progression	miR-3619-5p	CTNNB1	HCC	[47, 52, 94, 95]
	Promotes cancer progression	miR-4302	ZNF121	Lung cancer	
	Promotes cancer progression	miR-1261	C8orf4	PTC	
	Inhibits cancer progression	miR-130a/−107	PTEN	Gastric Cancer	
circTCF25	Promotes cancer progression	miR-103a-3p/− 107	CDK6	Bladder carcinoma	[49]
Hsa_circ_0008039 (circPRKAR1B)	Promote proliferation/migration	miR-432-5p	E2F3	Breast cancer cells	[51]
circITCH	Inhibits proliferation	miR-214	ITCH/CTNNB1	CRC	[53–55, 93]
	Inhibits cancer progression	miR-17/− 224	P21, PTEN	Bladder cancer	
	Inhibits cancer progression	miR-22-3p	CBL/CTNNB1	Papillary thyroid cancer	
	Inhibits cancer progression	miR-145	RASA1	Ovarian cancer	
Hsa_circ_0002052 (circPAPPA)	Inhibits cancer progression	miR-1205	APC2	Osteosarcoma	[56]
circMYLK	Promotes cancer progression	miR-29a	LAMC, TRAF4	PC-a	[58]
circRNA_0084043 (circADAM9)	Promotes cancer progression	miR-153-3p	SNAIL	melanoma	[59]
circIRAK3	Promote migration/invasion	miR-3607	FOXC1	Breast cancer cells	[60]
circNASP	Promotes cancer progression	miR-1253	FOXF1	Osteosarcoma	[61]
circMAN2B2	Promotes proliferation/migration	miR-1275	FOXK1	Lung cancer	[62]
circSHKBP1	Promotes angiogenesis	miR-544a/− 379	FOXP1, FOXP2	Endothelial cells	[63]

Table 1 circRNAs that function as miRNA sponges *(Continued)*

CircRNA	Biological functions	miRNA	Targets	Cell type	Ref
circSMAD2	Inhibits EMT	miR-629	TRIM33	HCC	[64]
circFOXO3	Inhibits cancer progression	miR-155	FOXO3	NSCLC	[65]
circFGFR4	Myoblast differentiation	miR-107	WNT3A	Bovine myoblast	[67]
circSVIL	Myoblast differentiation	miR-203	MEF2C, JUN	Chicken myoblast	[68]
circZC3H4	Macrophage activation	miR-212	ZC3H4	Alveolar macrophage	[75]
circHIPK2	Astrocyte activation ER stress	miR-124 miR-506-3p	SIGMAR1 SIGMAR1	Astrocyte HPF-a	[77, 115]
circHECTD1	Inhibits astrocyte activation	miR-142	TIPARP	Brain	[78]
circGRB10	Inhibits apoptosis	miR-328-5p	ERBB2	NP cells	[79]
Hsa_circ_0043256 (circACACA)	Induced apoptosis	miR-1252	ITCH	NSCLC	[80]
circDLGAP4	Ameliorates ischemic stroke	miR-143	HECTD1	Brain tissue	[82]
circVMA21	Against IDD	miR-200c	XIAP	NP cells	[83]
circZNF609	Retinal neurodegeneration Vascular dysfunction Myoblast differentiation	miR-615 miR-615 miR-194-5p	METRN MEF2A BCLAF1	RGC Vascular endothelial C2C12	[84, 85, 116]
circPVT1	Promotes proliferation	miR-497-5p	Aurka, Bub1, mKi67	HNSCC	[117]
circMTO1	Inhibits cancer progression	miR-9	P21	HCC	[118]
circITGA7	Inhibits proliferation/metastasis	miR-370-3p	NF1	CRC	[119]
circACTA2	VSMC contraction	miR-548f-5p	SMA	HASMC	[120]
circCCDC66	Promotes cancer progression	miR-33b/− 93/− 185	DNMT3B, MYC, EZH2,	CRC	[121]
circFBLIM1	Promotes cancer progression	miR-346	FBLIM1	HCC	[122]
circATP2B1	Promotes invasion	miR-204-3p	FN1	CCRCC	[123]
circLARP4	Inhibits proliferation/invasion	miR-424-5p	LATS1	Gastric cancer	[124]
Hsa_circ_0000799 (circBPTF)	Promotes cancer progression	miR-31-5p	RAB27A	Bladder cancer	[125]
circRNA_000203 (circMYO9A)	Promotes fibrosis	miR-26b-5p	Col1a2, Col3a1, SMA, CTGF	Cardiac fibroblast	[126]
circRNA_8924 (circC1orf116)	Promotes cancer progression	miR-518d-5p/− 519-5p	CBX8	Cervical cancer cells	[127]
circRNA_008913 (circADAT1)	Reduces carcinogenesis	miR-889	DAB2IP	HaCaT	[128]

Abbreviations: *CCRCC* (Clear Cell Renal Cell Carcinoma), *CRC* (Colorectal cancer), *ESCC* (Esophageal squamous cell carcinoma), *HASMC* (Human aortic smooth muscle cells), *HCC* (Hepatocellular carcinoma), *HNSCC* (Head and neck squamous cell carcinoma), *IDD* (intervertebral disc degeneration), *NP* cells (Nucleus pulposus cells), *NSCLC* (Non-small-cell lung carcinoma), *PDLSC* (Periodontal ligament stem cells), *PTC* (Papillary thyroid cancer), *RGC* (Retinal ganglion cells), *VSMC* (Vascular smooth muscle cells)

Table 2 CircRNAs that function as protein decoys

CircRNA	Biological functions	Interacting protein	Cell type	Ref
circFOXO3	Inhibits cell cycle progression Cardiac senescence Induces apoptosis	P21, CDK2 ID-1, E2F1, FAK, HIF1α MDM2, P53	Non-cancer cells Heart tissue Non-cancer cells	[96, 97, 129]
circANRIL	rRNA maturation	PES1	Vascular tissue	[81]
circHECTD1	Macrophage activation	ZC3H12A	Macrophage	[76]
circDNMT1	Promotes proliferation	P53, AUF1	Breast cancer cells	[98]
circSMARCA5	Tumor suppressor	SRSF1	Glioblastoma	[99]
circMTO1	Inhibits proliferation	TRAF4	Breast cancer cells	[100]
circAMOTL1	Promotes cell survival	PDK1, AKT1	Cardiomyocytes	[101]
circPABPN1	Suppresses PABPN1 translation	HuR	HeLa	[24]

detectable protein product in fly head by targeted mass spectrometry analysis of Mbl immunoprecipitate [29]. Importantly, Legnini et al. demonstrates that *circZNF609* regulates myogenesis and can be translated into peptides, suggesting that *circZNF609* may exert its function through protein expression [27]. Unfortunately, the phenotype could not be unequivocally linked to protein products because the re-expression of *circZNF609* by plasmids or naked RNA induces a non-specific block of myoblast proliferation. In addition, Zhang et al. finds that circular *lncRNA-PINT* can be translated into a small peptide to suppress glioblastoma cell proliferation; this action is mediated by trapping of PAF1c to inhibit translational elongation of oncogenes [30]. Moreover, some specific circRNAs are found to be associated with translating ribosomes in mammalian cells [29], which is highly suggestive that these circRNAs produce functional peptides.

The biogenesis of CircRNAs

CircRNAs are generated by back-splicing, wherein the 3′ terminus of a downstream exon is ligated to the 5′ terminus of an upstream exon. Similar to canonical splicing, back-splicing of circRNAs is tightly regulated by *cis*-elements (i.e., DNA sequences) and trans-factors (i.e., RNA binding proteins). Generally, most circRNAs contain two or three exons without intron segregation, while those circRNAs that contain only one exon typically exhibit a longer than average exon length [35]. Nevertheless, analysis of circular exon sequences and circular exon replacement assays both suggest that there are no specific exonic sequences that control circRNA formation [32]. On the other hand, the flanking introns of circRNAs are usually longer than average and enriched with complementary repeats [12, 35, 106]. Capel et al. are the first to show that complementary intronic sequences (CIS) flanked the circular *SRY* gene in mouse, suggesting that CIS may mediate the formation of this circRNA [5]. Further studies indicated that CIS are enriched in flanking introns of circRNAs from various species, including mouse, pig, *C. elegans* and *Drosophila* [32, 106–108]. Importantly, deletion of CIS adjacent to Laccase2 exon 2 abolishes circular laccase2 expression in *Drosophila* [38]. Furthermore, the primate-specific Alu repetitive elements, have been implicated in the biogenesis of some circRNAs. Jeck et al. first demonstrates that Alu repetitive elements are enriched in the flanking introns of human circRNAs [12], and Zhang et al. extends this observation, confirming that the pairing between Alu repetitive elements with reverse orientation regulates the expression of linear and circular isoforms [35, 109].

With regard to trans-factors in circRNA formation, Ashwal-Fluss et al. showed that the Mbl binding sites on flanking introns are necessary for circMbl formation; furthermore, ectopic expression of Mbl enhances circMbl expression, suggesting that the splicing factor was involved in circRNA biogenesis [32]. Conn et al. further demonstrates that the disruption of splicing factor QKI or its binding sites on flanking introns attenuates circRNA formation during EMT, and Errichelli et al. find that disruption of splicing factor FUS in mouse motor neurons affects circRNA expression [110]. Our group also show that splicing factor ESRP1 can promote circRNA expression through intronic binding sites flanking *circBIRC6* in human ESCs [36]. In addition to promoting circRNA biogenesis, splicing factors have also been shown to repress circRNA formation. For example, disruption of SR family members SRSF1/6/11 or hnRNP family member Hrb27C enhances circular laccase2 expression in *Drosophila* [38]. In addition, double-stranded RNA binding proteins, such as ILF3 (NF90/NF110) or DHX9, also have been shown to regulate circRNA formation. Disruption of ILF3 (NF90/NF110) downregulates circRNA expression, while disruption of DHX9 upregulates circRNA expression in human cells [111, 112]. The binding of ILF3 (NF90/NF110) is shown to stabilize CIS pairs, while the binding of DHX9 recruits ADAR1 to disrupt the pairing of Alu repetitive elements through A to I editing.

Conclusion

In this review, we survey the known functions of circRNAs in cell proliferation, EMT, development and other cellular processes. We also summarize the mechanisms by which circRNAs function, including RNA and protein interactions, and we describe the regulatory elements that are known to be involved in circRNA formation. Despite the broad range of findings regarding circRNA functions and regulation, many questions remain to be resolved. For example, how circRNAs are degraded in the cell and how degradation works in conjunction with biogenesis to respond to dynamic cellular states is an important territory that awaits further exploration. Some researchers have suggested that exocytosis may be an important pathway for circRNA clearance [113], but the selective enrichment of circRNAs in exosomes from different cell types argues against to this model [26]. Although the expression of circRNAs has been studied in the context of many human diseases, our understanding of the different roles in normal physiology and the disease conditions is limited for the vast majority of identified circRNAs. Finally, improvements in technology to detect circRNAs at a single-cell level and methods to efficiently manipulate circRNAs without affecting linear cognates will be key for gaining further insights into the functions of circRNAs and the mechanisms underpinning their regulatory roles.

Abbreviations

CCRCC: Clear Cell Renal Cell Carcinoma; CircRNAs: Circular RNA; CIS: Complementary intronic sequences; CRC: Colorectal cancer; EMT: Epithelial-mesenchymal transition; ESCC: Esophageal squamous cell carcinoma; HASMC: Human aortic smooth muscle cells; HCC: Hepatocellular carcinoma; HNSCC: Head and neck squamous cell carcinoma; IDD: Intervertebral disc degeneration; IRES: Internal ribosome entry site; LncRNAs: Long non-coding RNAs; miRNAs: micro RNAs; NP cells: Nucleus pulposus cells; NSCLC: Non-small-cell lung carcinoma; PDLSC: Periodontal ligament stem cells; PTC: Papillary thyroid cancer; RGC: Retinal ganglion cells; VSMC: Vascular smooth muscle cells

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